Exploring the Role of the foraging Gene on Egg-Laying Preferences

in Drosophila melanogaster

by

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**Abstract**

Egg-laying decisions can have significant fitness consequences. In female *Drosophila melanogaster*, egg-laying involves foraging-like behaviour. Natural allelic variation in *foraging* (*for*) underlies the rover/sitter foraging behaviour polymorphism found in *D. melanogaster*. *for* encodes a cGMP-dependent protein kinase (PKG) where rovers have higher *for*-PKG transcript levels and PKG activity than sitters. Interestingly, the orthologue of *for* in nematodes (*egl-4*) affects both egg-laying and foraging behaviours. When given a choice between low- and high-nutrient patches, rovers preferentially lay more eggs on the low-nutrient patches while sitters and a sitter mutant prefer high-nutrient patches. Using the neuronal driver *elav*-GAL4, rover-like preferences were rescued in sitter flies. Compared to sitters, rovers have higher fitness on a sub-optimal substrate which may explain the observed egg-laying preferences. By studying the link from genes to behaviour, this study provides insight to the evolutionary basis and maintenance of behaviour.
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Dedication

For Grandpa and Grandma McC

&

Grandpa and Grandma McG
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1. Introduction

Behaviours are important to the survival and fitness of many organisms. These behaviours include locating food, catching prey, defending territories, finding potential mates, courting and successfully mating, finding and building a home, finding a location to build a nest or deposit eggs, and the parental care of offspring (Alcock 2005). Behaviours are very complex traits that are highly influenced by both the environment and a network of underlying genes (Sokolowski 2001; Bucan and Abel 2002; Mackay and Anholt 2006; Kent et al. 2008). Many analyses often utilize quantitative genetic measures such as heritability to assess the relative roles of genes and environment on behaviour. Examples include alternative mating strategies (Cade 1981), calling song in crickets (Gray and Cade 1999), courtship song in moths (Collins et al. 1999), and fruit flies (Aspi and Koikkala 1993; Ritchie and Kyriacou 1996), flight dispersal rate in butterflies (Haag et al. 2005), foraging in birds (Lemon 1993), life history tactics in fish (Theriault et al. 2007), migratory tendency in grasshoppers (Kent and Rankin 2001), signalling time in crickets (Bertram et al. 2007) and predatory foraging behaviour in mites (Nachappa et al. 2010). However, in many cases the particular genes underlying behaviour have been identified (reviewed in Sokolowski 2001; Bucan and Abel 2002; Rankin 2002) and this list of genes is increasing rapidly as more researchers are exploring the links between genes and behaviour (see Fitzpatrick et al. 2005; Anholt and Mackay 2004; Mackay and Anholt 2007; Robinson et al. 2008). The identification of the genes that affect behaviour allows for: i) manipulating behaviour via altering gene expression and/or protein activity, ii) identifying and classifying individuals to behavioural classes without having to measure behaviour, iii) gaining a better understanding of the evolution of the behaviour by studying the sequences of the genes both within a species and
across other taxa. Moreover, work in behaviour genetics has begun to integrate mechanistic studies with those concerning evolution and ecology.

Around the same time that Mendel published his work on heritable traits in pea plants, Francis Galton published what is thought to be the first quantitative genetic study tracing behavioural traits (reviewed in Greenspan 2004; 2008). Sometime later, Jerry Hirsch began his classic work on understanding whether genes influenced geotactic behaviour, the tendency to travel either up or down in a vertical maze. Hirsch and Erlenmeyer-Kimling (1961; 1962) showed a significant role of genes on geotactic tendencies by artificially selecting two groups of flies – those that consistently go up in the maze and those that consistently go down – all from a standard position with no up or down preference. This work was highly influential as it laid the foundation for one main avenue of behavioural genetics research – finding extremes of a normally distributed behaviour, isolating individual’s displaying these extremes and studying the underlying genes. This is often referred to as the ‘natural variant’ or ‘top-down’ approach. In 1967, Seymour Benzer introduced the emerging field of behaviour genetics to a very different avenue often referred to as the ‘single-gene mutant’ or ‘bottom-up’ approach.’ Benzer (1967) mutated normal or ‘wild-type’ individuals and screened for changes in phototaxis, movement in response to light. The single-gene mutant approach facilitates the identification if individual genes whereas the natural variants approach rarely extended beyond artificially selected lines and heritability estimates.

Although the single-gene mutant approach is a valuable and rapid method for understanding the link between genes and behaviour, this ‘bottom-up’ method has received much criticism, especially from an ecological point of view (Boake et al. 2002; Greenspan 2004; Sokolowski 2001). These laboratory-induced mutants often show extreme disruptions in
behaviour and development and thus they are unlikely to survive under natural conditions. However, recent evidence suggests that the same genes localized using the ‘bottom-up’ mutant approach often differ in nature, with more subtle variations than those produced in the laboratory. For example, the *period* gene (*per*), a circadian regulator that was initially identified by mutation (Konopka and Benzer 1971), has now been shown to have natural variants along a latitudinal cline (Sawyer *et al.* 1997) and *timeless (tim)*, another circadian gene identified by mutation analyses (Sehgal *et al.* 1994), was recently shown to affect natural variation in diapause in European *Drosophila* populations (Sandrelli *et al.* 2007; Tauber *et al.* 2007). Therefore, although initial scepticisms remain valid, evolutionary ecologists are now appreciating the information gained from ‘bottom up’ methods (Fitzpatrick *et al.* 2005 and Boake *et al.* 2002).

Currently, most of the genes influencing behaviour have been identified in genetic model organisms – mainly the fruit fly, nematode worm, and mouse. However, based on the ever-growing evidence showing a conservation in gene sequence and function across evolutionary time (e.g. Hox genes; Ruddle *et al.* 1994), a candidate gene approach taking information from the aforementioned model organisms and extending them to additional organisms is proving to be a very successful avenue of study (Fitzpatrick *et al.* 2005). A superb example of this conservation of gene function influencing behaviour comes again from the *period* gene discussed above. In addition to *per*’s effects on circadian rhythms and locomotor activities in fruit flies, this same gene has been implicated in similar phenotypes in house flies (Piccin *et al.* 2000), tephritid flies (An *et al.* 2002), honey bees (Bloch *et al.* 2001; Toma *et al.* 2000), moths (Levine *et al.* 1995), lancelets (Schomerus *et al.* 2008), mice (Zheng *et al.*1999) and humans (Kloss *et al.* 1998; Toh *et al.* 2001). Furthermore, a landmark study by Levine and colleagues (1995) transferred the *per* gene sequence from the silkmoth, *Antheraea pernyi*, into a fruit fly,
**Drosophila melanogaster**, that contained a knocked out *per* gene. The authors found that this transfer was sufficient to restore circadian function in the fly. This conservation in gene function is important for: i) understanding the evolutionary genetics of behaviour, and ii) opening the door for studying the particular genes that affect behaviour in a wide array of taxa.

An excellent model system for the study of genes and behaviour comes from the fruit fly *Drosophila melanogaster*. This study system integrates both the ‘top-down’ and ‘bottom-up’ approaches to gain a better understanding of the genes involved in food search and feeding behaviours (i.e. foraging). In 1980, Sokolowski discovered a polymorphism in larval foraging patterns. Later, this polymorphism was attributed to two naturally existing alleles of the *foraging* gene (*for*): *for^R* (rover) and *for^s* (sitter) (de Belle et al. 1989). Rover larvae and adults both show increased locomotion when feeding and searching for new food sources compared to sitter individuals (Sokolowski 1980; Pereira and Sokolowski 1993). With respect to larval foraging, the *for^R* allele is genetically dominant to *for^s*. Allelic variation is maintained in the population by negative frequency-dependent selection (Fitzpatrick et al. 2007) and rover/sitter frequencies can be shifted by density-dependent selection (Sokolowski et al. 1997). In 1997, Osborne et al. cloned the *foraging* (*for*) gene where it was discovered that it encodes a cGMP-dependent protein kinase or PKG and that rovers have increased mRNA transcript levels and increased PKG activity compared to sitters. A few years previous to this, the *for* locus was identified as a PKG-encoding region and was named *dg2* (Kalderon and Rubin 1989), thus *for* and *dg2* are synonymous. PKG functions by phosphorylating serine and threonine residues on a diverse array of proteins (Edelman et al. 1987). PKG is responsible for major cellular processes such as signal transduction, muscle relaxation, nociception, and platelet function (Feil et al. 2005; Hofmann et al. 2006).
Genes often serve multiple roles within an organism and these multiple phenotypes are referred to as the pleiotropic effects of the gene (Caspari 1952; Barton 1990). In fact, many estimates suggest a majority of the genome harbours genes with pleiotropic effects (Caspari 1952; Fitzpatrick 2004) and this is also true for genes underlying behaviour (Sokolowski 2001). In addition to the role of for in larval foraging behaviour, natural allelic variation in for has also been attributed to variation in food intake/energy homeostasis (Kaun et al. 2007a), learning and memory (larvae: Kaun et al. 2007b, adults: Mery et al. 2007), sucrose responsiveness (Scheiner et al. 2004; Belay et al. 2007), temperature stress tolerance (Dawson-Scully et al. 2007) and anoxic stress tolerance (Dawson-Scully et al. 2010) and pupation site selection (Sokolowski 1985, Rodriguez et al. 1992). Moreover, accumulating evidence is showing that like per, for may have a conserved role in a variety of organisms. for/PKG has been implicated in the transition from nurse to forager in the honey bee (Apis mellifera, Ben-Shahar et al. 2002), foraging and caste determination in two ant species (Pogonomyrmex barbatus, Ingram et al. 2004 and Pheidole pallidula, Lucas and Sokolowski 2009), and necromenic host detection (Pristionchus pacificus, Hong et al. 2008) and food-dependent locomotion (Caenorhabditis elegans, Fujiwara et al. 2002) in nematode worms.

In C. elegans, the gene egl-4 (orthologous to for) is attributed to ‘roaming’ or ‘dwelling’ locomotory behaviours seen in the presence of food (Fujiwara et al. 2002). As with the fruit fly, egl-4 is associated with several pleiotropic phenotypes including food intake (You et al. 2008), sleep (Raizen et al. 2008), body size (Daniels et al. 2000; Fujiwara et al. 2002; Hirose et al. 2003), temperature stress (Yamada and Ohshima 2003), longevity (Hirose et al. 2003), olfactory adaptation (L’Etoile et al. 2002), dauer formation (i.e. diapause) (Daniels et al. 2000), and synaptic transmission (Daniels et al. 2000). Initially identified as an egg-laying mutant (Trent et
al. 1983), research on the role of egl-4 in egg-laying has been sparsely studied since that time. Linking back to flies, early work from Sokolowski (1981) gave some evidence of rover/sitter differences in oviposition. This potential shared pleiotropic effect between for in flies and egl-4 in worms on egg-laying and oviposition currently remains an important but open question.

The selection of a suitable location for oviposition (OSS, oviposition site selection) can have measurable implications on the development and survival of offspring. Evidence suggests that preferred oviposition substrates include those of high resource quality and enemy-free spaces (Jaenike 1978; Mangel 1989; Thompson 1991). In Drosophila spp. OSS has been shown to be influenced by many factors including alcohol content (Parsons and Kling 1977; Richmond and Gerking 1979), inherited preferences (Takamura 1980), geotactic tendencies (Pyle 1976), previously occupied food sources (del Solar and Palomino 1966), food texture (Chess and Ringo 1985) and the availability of novel substrates (Sheeba et al. 1998). More recently, Yang et al. (2008) show D. melanogaster flies prefer to oviposit on substrates with lower sugar concentrations. Yang et al. (2008) also give evidence that females probe each site with her proboscis and lay eggs on an egg-by-egg basis (i.e. eggs are not laid in batches), suggesting that females are constantly assessing their surroundings for suitable oviposition substrates and appear to do so using foraging-like behaviour. This is similar to studies using Lepidoptera, where foraging and oviposition have been shown to be linked (Janz et al. 2005; reviewed in Renwick 1994). However, despite a large amount of work on OSS, the genetic basis of oviposition preferences are still relatively unknown.

This thesis explores the role of for in the oviposition preferences of the fruit fly D. melanogaster based on two important observations: i) a candidate gene approach suggests an influence of for on oviposition since OSS in D. melanogaster involves a foraging-like behaviour
females locate food patches, assess patch quality using their proboscis (i.e. feed), and if suitable, deposit an egg), and ii) given the apparent conserved function of *for* and the similarities found in the pleiotropic effects of *for/egl*-4 in flies and worms, extending the egg-laying role of *egl*-4 to from worms to flies is intuitive. I explore this link by: 1) assessing the differences between rovers, sitters and the sitter mutant, (*for*) in egg-laying behaviour, 2) using a transgenic manipulation of *for* to increase the expression of *for/PKG*. My thesis also assesses the fitness costs and benefits stemming from egg-laying decisions by, 3) assessing the egg-laying behaviour of rovers, sitters and the sitter mutant when in the presence of previously laid eggs, and 4) assessing the rates of rover and sitter pupation when reared on differing quality diets in genotypic isolation as well as when competing with the alternative phenotype on the same substrate.

Yang *et al.* (2008) demonstrated that the sweetness of the substrate is important in the decision of where to deposit one's eggs in a wild-type *D. melanogaster* fly stock. Previous studies using the rover/sitter polymorphism have demonstrated clear differences in the sucrose responsiveness between rover and sitter flies (Scheiner *et al.* 2004; Belay *et al.* 2007), where rovers are able to detect lower levels of sucrose then sitters as demonstrated by a proboscis extension response. It is predicted that differences in sucrose levels between possible egg-laying substrates will lead to differences in rover and sitter egg-laying preferences, however the direction of these preferences are not known.

By assessing the role of *for* in oviposition and the fitness consequences resulting from egg-laying preferences, this thesis aims to: i) provide empirical evidence for the genetic basis of a complex behaviour (OSS), and ii) attempts to understand the maintenance of a polymorphic trait. To answer these questions, this thesis combines elements of behavioural ecology and
genetics, two traditionally separate research avenues that when combined form the framework for understanding the evolution of species and the maintenance of traits.
2. Materials and Methods

This thesis is organized into four experiments. General methods applying to all experiments are first described below and are followed by methods specific to the various experiments.

Experiment 1: Does for affect oviposition preferences?
Experiment 2: Confirming the role of for in oviposition preferences by transgenic manipulation
Experiment 3: Does the presence of previously laid eggs affect rover/sitter preferences?
Experiment 4: Assessing fitness consequences associated with egg-laying decisions.

General Methods

Fly Stocks.

Rover and sitter strains are homozygous for the for\(^R\) and for\(^s\) alleles, respectively, on the second chromosomes and share co-isogenic third chromosomes from the for\(^R\) strain (Sokolowski 1980). for\(^s2\) is a sitter-mutant strain that was generated on a rover genetic background, but with an induced mutation at the foraging locus which confers sitter-like for mRNA-expression, PKG activity levels, and behaviour (de Belle et al. 1993; Pereira and Sokolowski 1993; Osborne et al. 1997). The for\(^s2\) mutant strain provides an important second chromosome control since the sitter-like foraging behaviour of this strain is linked to the induced mutation in the for gene (see Pereira and Sokolowski 1993, de Belle et al. 1993). Consequently, any phenotypic differences between for\(^s2\) and for\(^R\) can be directly attributed to for. For the transgenic experiments, several strains were used: w\(^1\); for\(^s\); elav-GAL4, w\(^1\); for\(^s\); UAS-for\(^T2\), and w\(^1\); for\(^s\); +. The specific crosses used in the transgenic experiments are outlined and described below. To test fitness of genotypes
when combined, I needed an easy phenotypic marker to identify rovers from sitters. For this I used homozygous rover and sitter flies that had been marked with green fluorescent protein (GFP). These GFP-marked rover and sitter flies have a substituted pair of third chromosomes containing the GFP marker but otherwise are identical to their rover/sitter counterparts. This marker allows for easy comparison of eggs, larvae and pupae and it has been found that foraging path lengths of GFP-marked rovers and sitters do not differ from unmarked rovers and sitters (Fitzpatrick et al. 2007). Therefore, fly lines used in this experiment were +; for\textsuperscript{R}; +, +; for\textsuperscript{s}; +, +; for\textsuperscript{R}; Ubi-GFP, and +; for\textsuperscript{s}; Ubi-GFP.

**Fly Rearing Conditions and Media**

Fly stocks were maintained in 170 mL plastic culture bottles with 40 mL of standard culture medium and were reared at 23 ± 1°C, and 65 ± 5 % relative humidity, and a 12L:12D photocycle (lights off at 1900 h). One litre of standard culture medium contains 50 g of Baker’s yeast (Fleischmann’s), 100 g of sucrose, 16 g of agar, 0.1 g of KH\textsubscript{2}PO\textsubscript{4}, 8 g of C\textsubscript{6}H\textsubscript{4}KNaO\textsubscript{6}, 0.5 g of NaCl, 0.5 g MgCl\textsubscript{2}, 0.5g Fe\textsubscript{2}(SO\textsubscript{4})\textsubscript{3} and 5 mL C\textsubscript{3}H\textsubscript{6}O\textsubscript{2} (propionic acid).

**Nutrient-Adjusted Food Patches for Oviposition**

Recent work on oviposition in *D. melanogaster* has reported that common laboratory wild-type strains prefer to oviposit on substrates that are low in sugar content (Yang et al. 2008). Following this, a simple assay was devised that allows for groups of similarly aged gravid females to lay on an array of food patches containing either high-nutrient food (i.e. standard rearing conditions) or low-nutrient food (85% reduction in yeast and sugar) (see Fitzpatrick et al. 2007 for a detailed description of the media). Media used in experimental trials (low- and high-
nutrient) was darkened using charcoal (2.6 g/L). Charcoal is non-nutritive and has been used to darken food to increase contrast between media and larvae (see Fitzpatrick and Szewczyk 2005). In this study, charcoal was used to facilitate egg counting. Experimental media was made in 1 L batches, poured into plastic vials (25 × 95 mm, Fisher Scientific Canada) and stored at 4 °C until day of use. Each day, the number of microcentrifuge caps filled with medium was determined by the number of trials being put into the incubator that night. To prepare caps, medium was transferred from the vial to a beaker, then placed in a microwave, and melted for 20 seconds. Media was then poured into caps using a 12 mL syringe. The high-nutrient food represents the standard culture media described above whereas the low-nutrient food contains an 85% reduction in the yeast and sugar components of the standard media.

Experimental Arena & Setup

Oviposition arenas consisted of plastic storage containers (11 cm × 11 cm × 5.5 cm, Dollarama). A sponge was placed in a hole cut in lid to allow for sufficient air exchange and the transferring of flies into the container. The food patches for oviposition consisted of microcentrifuge tube caps (1.5 mL) filled with either the high- or low-nutrient food. Each trial used four food caps (2 high-nutrient: 2 low-nutrient) that were placed randomly (coin flip) in one of four corners of the testing arena and secured by plastiscine. For each individual trial, either the low- or the high-nutrient caps were marked with a dot from a black felt pen (Sharpie) and this was determined randomly by a coin flip. Between 15:30 and 16:00 h 64 mated females were placed into the testing arenas which were then immediately placed in the incubator 25 ± 1 °C and 65 ± 5 % relative humidity. Flies were kept in the incubator overnight since the majority of egg-laying occurs in the evening hours (Ashburner 1989). The following morning, the females were
removed from the arenas by CO₂ anaesthetization. The oviposition caps were then removed and the number of eggs in each were counted under a light microscope at 1.6 × magnification and recorded.

**Experiment-Specific Methods**

*Experiment 1: Does for affect oviposition preferences?*

*Isolating Flies for Oviposition Experiments*

  Founding flies (rover, sitter, sitter mutant) were removed from the stock bottles prior to the eclosion of their offspring. Each day thereafter, newly emerged adult flies were removed from the stock bottles and placed in new vials, labelled ‘mix’ and dated but kept genetically isolated. The following day, flies from the ‘mix’ vials were divided into sub-populations of 64 females and 20 males and placed in new vials until one day prior to the experiment. The establishment of these sub populations serves two purposes: i) it facilitates the preparation for one oviposition assay (64 females), and ii) fewer flies and a 3:1 ratio of females to males is ideal to ensure mating without excessive stressing of females by males. Males were removed from the subpopulations the following day. All females were 3 day old adults when assayed for oviposition preferences and all tests were done in genetic isolation.

*Experiment 2: Confirming the role of for in oviposition preferences by transgenic manipulation*

*Transgenic Crosses*

  The extensive use of *D. melanogaster* as a tool for studying genetics and developmental biology has led to the creation of numerous tools including the generation of transgenic fly stocks. By capitalizing on a molecular interaction found in yeast (the binding of the GAL4
protein to the UAS sequence), fly researchers have developed the ability to target the expression of specific genes to specific locations and tissues (Brand and Perrimon 1993). Such fly stocks are available to transgenically over express for-mRNA in sitters to induce rover-like behaviour. As mentioned, rovers have higher PKG activity than sitters. By increasing PKG activity, rover-like food-related behaviours have been rescued in sitter flies (Belay et al. 2007; Kaun et al. 2007a; Kaun et al. 2007b; Mery et al. 2007; Dawson-Scully et al. 2007; Dawson-Scully et al. 2010). In this study we used the pan-neuronal GAL4 driver elav to up-regulate forT2 RNA in sitter flies leading to rover-like PKG activity. Transgenic flies used in this experiment were previously generated by crossing viable homozygous P-element insertion strains (UAS-forT2) into a for^8 genetic background, creating the stock w^1; for^8; UAS-forT2 (Belay et al. 2007). In a separate strain, the transgenic GAL4 driver line was crossed into a sitter genetic background to produce the following: w^1; for^8; elav-GAL4. The elav-GAL4 transgene is a pan-neuronal driver which, when heterozygous with UAS-forT2 leads to increased expression of for-mRNA in the nervous system (Belay et al. 2007). When the elav-GAL4 and UAS-forT2 elements are combined in the same fly (details below) they result in an increase in for-mRNA and PKG levels similar to that of rovers (Belay et al. 2007). A cross was made between female w^1; for^8; UAS-forT2 and male w^1; for^8; elav-GAL4. This produced flies with the following genotype: w^1; for^8; UAS-forT2/ elav-GAL4. To control for the transgenic insertions while also accounting for copy number, two additional control crosses were performed: 1) female w^1; for^8; UAS-forT2 were crossed to male w^1; for^8; + to produce the following progeny: w^1; for^8; UAS-forT2/ +, and 2) female w^1; for^8; + were crossed to male w^1; for^8; elav-GAL4 to produce w^1; for^8; elav-GAL4/ +. Oviposition assays (as described in experiment 1) were then conducted at 25 °C on the following flies: w^1; for^8;
UAS-forT2 / elav-GAL4 (overexpression), w¹; for⁸; UAS-forT2 / + (control 1), and w¹; for⁸; elav-GAL4/ + (control 2).

**Experiment 3: Does the presences of previously laid eggs affect rover/sitter preferences?**

To test for an effect of the presence of another genotype’s eggs on the egg-laying preferences of rovers and sitters, I pre-seeded oviposition food patches with rover or sitter eggs in a rover or sitter pattern. The pre-seeded eggs used were either +; for⁸; Ubi-GFP or +; for⁸; Ubi-GFP to facilitate ease of differentiation between genotypes. Eggs were obtained by allowing females to oviposit on a grape juice-agar medium (grape plates) overnight at 25 ± 1 °C, 65 ± 5 % relative humidity (Pereira et al. 1995). The next morning, adults were removed and grape plates (with eggs) were placed in a fridge at 4 °C. The following day, between 13:00 and 14:30, 40 eggs were transferred to experimental food caps using a probe. Four treatments were tested using all four possible combinations of egg genotype (rover/sitter) and egg-laying pattern (rover/sitter). Egg-laying pattern was established by rover/sitter egg-laying proportions laid on high- and low-nutrient food seen in Figure 1 where a density of 40 eggs was maintained across all combinations. Prior to the introduction of the flies, the food caps were pre-seeded depending on the treatment conditions. Given that rovers lay approximately 65% of their eggs on low quality food and sitters lay approximately 65% of their eggs on high quality food (see results), food caps of the same nutrient quality either received 26 rover or sitter eggs, whereas the other two food caps were pre-seeded with 14 rover or sitter eggs (Table 1.). Pre-seeded rover/sitter eggs were distributed evenly amongst their assigned food caps. Following this pre-seeding step the oviposition assay was carried out as described above.
**Experiment 4: Assessing fitness consequences associated with egg-laying decisions.**

**Competition Experimental Setup**

Competition fly vials (50 mL) were prepared with 6 mL of charcoal free high- or low-nutrient food. Larvae were collected by transferring adult flies into bottles with a grape plate upon which to oviposit at 14:00. These bottles were then placed in an incubator (25 ± 1°C, 65 ± 5 % relative humidity) overnight. To guarantee similar age of all hatched larvae, at 09:00 the following morning, the grape-agar medium was removed from the fly bottles and all hatched first instar larvae were removed. The grape plates were returned to the incubator (25 ± 1°C) for later larval collection (see Pereira et al. 1995)

**Seeding competition vials**

At 14:00, all grape plates were removed from the incubator. Previous work has shown that 32 larvae per 6 mL vial are sufficient to cause significant competitive stress (Fitzpatrick et al 2007). Therefore, the ratio of larvae seeded into each vial was calculated out of a standard density of 32. Using the egg-laying preferences of rovers and sitters and including the increase in fecundity observed in rovers, this was calculated to be 84% rover larvae to 16% sitter larvae which translates to 27 rover larvae and 5 sitter larvae. I then devised four treatments for both low- and high-nutrient food to assess the effect of the observed egg-laying patterns on survivability to pupae. Treatments were as follows (see also Table 2):

i) 27 +;for\(^K\);Ubi-GFP (rover-GFP) : 5 +;for\(^R\);+ (sitter)

ii) 5 +;for\(^R\);Ubi-GFP (sitter-GFP) : 27 +;for\(^K\);+ (rover)

iii) 5 +;for\(^R\);Ubi-GFP (rover-GFP) : 27 +;for\(^R\);+ (sitter)

iv) 27 +;for\(^R\);Ubi-GFP (sitter-GFP) : 5 +;for\(^K\);+ (rover)
Control treatments were also assayed. Here the vials were seeded with either 32 rovers or 32 sitters to control for any genotype effects. All vials were seeded over two days in which the above collecting method was repeated for trials on the second day.

Data Collection

Each day, all seeded vials were removed from the incubator and the number of pupae within each vial was counted and recorded from day 4 to 14 days after hatching. GFP-marked individuals were counted using a stereoscope affixed with a GFP attachment.

Statistical Analyses

I used arcsine square root transformations to normalize all data for statistical analyses involving proportions (e.g. egg-laying preferences and fitness) (Zar 1999). Normality was assessed using Wilk-Shapiro statistics and since there were no significant deviations from normality I proceeded with parametric ANOVA. In the experiment comparing the proportion of eggs laid on low-nutrient substrate of rovers, sitters and the sitter mutant (Figure 1.), I used a One-Way ANOVA to test the main effects of genotype on egg-laying preferences. I conducted a similar One-Way ANOVA on preferences for low-nutrient substrate when assessing proportion of eggs laid in the transgenic manipulation experiment (Figure 2). In the pre-seeded egg experiment (Figure 3), I conducted a Three-Way ANOVA to test for main effects of ovipositing genotype (rover, sitter, sitter mutant), pre-seeded egg type (rover, sitter), and pre-seeded pattern (rover, sitter) and all possible interactions. In the fitness experiment (Figure 4) I assessed differences in survival of the control groups using a Two-Way ANOVA testing main effects of genotype (rover, sitter) and nutrient abundance (low- and high-nutrient), and a genotype ×
nutrient abundance interaction on fitness. In the competition treatments (Figure 5) under low-nutrient conditions, I conducted a Two-Way ANOVA testing main effects of genotype (rover, sitter) and ratio (84:16 and 16:84 of rover: sitter). All statistical tests were performed using JMP 8.0.
3. Results

The role of for in oviposition site selection in Drosophila melanogaster

I assayed the oviposition preferences of rovers (for$^R$), sitters (for$^S$) and sitter mutant (for$^{S2}$) flies when given a choice between high- and low-nutrient substrates. When assayed for egg-laying preferences, I found that rovers lay a significantly higher proportion of eggs (0.65 ± 0.02, mean ± s.e.m., N = 40 trials) on low-nutrient food than sitters (0.31 ± 0.03, N = 40 trials) and the sitter mutant (0.34 ± 0.02, N = 40 trials) (Figure 1; One-Way ANOVA, F$_{2,117}$ = 56.30, p < 0.0001, Tukey post hoc). The total output of egg-laying was different for the three genotypes we assayed. I found that rovers lay significantly more eggs per trial (66.25 ± 5.03, N = 40 trials) than either sitters (23.6 ± 3.00, N = 40 trials) or the sitter mutant (32.2 ± 2.54, N = 40 trials) (One-Way ANOVA, F$_{2,117}$ = 37.43, p < 0.0001, Tukey post hoc).

Rovers have higher for-mRNA expression levels and PKG activity levels than sitters (Osborne et al. 1997). Previous work has shown that transgenically increasing for gene expression in sitters to a level similar to rovers also leads to rover-like PKG activity levels and behaviour (Osborne et al. 1997; Belay et al. 2007; Kaun et al. 2007a,b; Mery et al. 2007). In this study I used the pan-neuronal driver elav-GAL4 to transgenically increase the expression for in sitter flies leading to rover-like mRNA levels and PKG activity levels (see Methods). When assayed for egg-laying preference, I found that the experimental flies (w$^1$; for$^S$; elav-GAL4/UAS-forT2) showed rover-like oviposition preferences by laying a significantly greater proportion of their eggs on the low-nutrient food (0.81 ± 0.07, N = 21 trials), than either of the experimental controls: w$^1$; for$^S$; elav-GAL4/+ (0.40 ± 0.03, N = 21 trials) or w$^1$; for$^S$; +/UAS-forT2 (0.40 ± 0.05, N = 21 trials) (Figure 2; One-Way ANOVA F$_{2,60}$ = 16.73, p < 0.0001, Tukey
post hoc). I also assessed egg-laying numbers across genotypes and found that the elav-GAL4 control cross laid significantly more eggs (18.81 ± 4.02 eggs, N = 21) than the experimental GAL4/UAS cross (5.81 ± 2.12, N = 21) and the UAS- forT2 control cross (7.33 ± 1.12, N = 21) (One-Way ANOVA, F$_{2,60}$ = 6.12, p < 0.002, Tukey post hoc).

**Fitness consequences of rover and sitter egg-laying preferences**

In the experiments previously described, I have assessed rover and sitter egg-laying preferences in genetic isolation. However, in nature, rovers and sitters coexist and interact. In an attempt to assess the fitness consequences of egg-laying decisions in a semi-natural scenario, I first attempted to have rovers compete with sitters. By using GFP-tagged flies I conducted oviposition assays for sub-populations with individuals of both genotypes. After which, the oviposition food caps from the trials were to be transferred to a vial containing an abundance of fly media upon which eggs could hatch and larvae develop. However, as the experiment progressed, a drastic reduction in fecundity in the GFP strains became apparent (see Appendix 5). To overcome this reduction in fecundity, I needed to modify my experimental design. My first set of experiments assessed egg-laying preferences when rover and sitters encountered substrates that contained eggs to simulate previous egg-laying by a conspecific. To do this I pre-seeded food caps with either rover or sitter eggs distributed in either a ‘rover-like’ or ‘sitter-like’ oviposition pattern (see above). This scenario has potential relevance to the natural world since flies are known to prefer oviposition sites that have been previously occupied (del Solar and Palomino 1966). Pre-seeded eggs were harvested from rovers and sitters carrying the GFP marker (Fitzpatrick et al. 2007). This design allowed me to circumvent the fecundity issues in the GFP lines while still making use of the GFP marker to differentiate between pre-seeded and
experimental eggs. My analysis considered ovipositing genotype (rover, sitter, sitter mutant), pre-seeded eggs (rover, sitter), and pre-seeded pattern (rover, sitter). I conducted a Three-Way ANOVA that considered the main factors listed above and all possible interactions where I failed to detect a significant effect for all variables and interactions except for the main effect of ovipositing genotype. Consequently, I reduced the model to a One-Way ANOVA looking at the effect of ovipositing genotype and found that rovers, across all treatments, laid a significantly greater proportion of their eggs on low-nutrient food than sitters and the sitter mutant (Figure 3; One-Way ANOVA, $F_{2,237} = 91.71$, $p < 0.0001$, Tukey post hoc). This supports the previous findings reported above (Figure 1). Given the differences in fecundity between rovers and sitters noted above, I conducted a Three-Way ANOVA using the total number of eggs as the independent variable ($F_{11,228} = 18.70$, $p < 0.0001$) and found significant main effects both pre-seeded egg type ($p = 0.0394$) and ovipositing genotype ($p < 0.0001$). All interactions were not statistically significant although the three-way interaction of ovipositing genotype $\times$ pre-seeded egg type $\times$ pre-seeded pattern was marginally non significant ($p = 0.06$). Following this I removed all interactions along with the main effect of pattern and reduced the model to a Two-Way ANOVA looking at the main effects of ovipositing genotype and pre-seeded egg type. I found that rovers laid significantly more eggs ($80.46 \pm 5.24$ eggs, $N = 80$ trials) than sitters ($23.31 \pm 1.7$ eggs, $N = 80$ trials) and the sitter mutant ($30.01 \pm 1.77$ eggs, $N = 80$ trials) (Two-Way ANOVA, $F_{2,236} = 88.55$, $p < 0.0001$). I also found that all genotypes laid marginally significantly more eggs when in the presence of sitter eggs (rover: $89.68 \pm 8.75$ eggs, $N = 40$, sitter: $22.83 \pm 2.68$ eggs, $N = 40$ and sitter mutant: $32.96 \pm 2.81$ eggs, $N = 40$, respectively) than when in the presence of rover eggs (rover: $71.25 \pm 5.50$ eggs, $N = 40$, sitter: $23.80 \pm 2.19$ eggs, $N = 40$ and sitter mutant: $27.05 \pm 2.07$ eggs, $N = 40$, respectively) (Two-Way ANOVA, $F_{1,236} =$
4.13, p = 0.04). However, this marginally significant effect of pre-seeding egg type does not survive a Bonferroni correction that accounts for multiple comparisons where the critical $\alpha$ value is revised to 0.025 (i.e. 0.05 / 2).

Given that rover, sitter and the sitter mutant egg-laying preferences are unaffected by the presence of previously laid eggs, this suggests that the preferences I found for low- and high-nutrient substrates in our isolated genotype experiments (see Figure 1) would be maintained in a natural setting where the genotypes coexist. Fitzpatrick et al. (2007) found that larval competition under low-nutrient conditions can lead to differential survival of rovers and sitters. However, this difference was not observed in higher-nutrient environments. To test for fitness effects of egg-laying preferences, I decided to have rover and sitter larvae compete in ratios that reflect their oviposition preferences in low-nutrient conditions by accounting for both preference and fecundity. I determined that although rovers lay 65% of their eggs on low-nutrient media compared to 31% by sitters, rovers laid 2.8 times as many eggs as sitters. Therefore, of eggs laid on low-nutrient food approximately 85% would be rover and 15% would be sitter. To maintain similar conditions with Fitzpatrick et al. (2007), I decided to have 32 larvae compete in vials containing 6 mL of low-nutrient media. As such, 27 individuals of one genotype (i.e. rovers) (84%) competed with 5 individuals of the other (i.e. sitters) (16%) to approximate their oviposition preferences mentioned above. To discern the genotypes within a competition vial I utilized the GFP marker strains described above. Experiments were conducted that competed GFP-rovers with unmarked sitters and GFP-sitters with unmarked rovers. Since first instar larvae are seeded into the competition vial, the reduced fecundity of the GFP strains mentioned above is circumvented. Initially, I had planned on assessing the fitness of rovers and sitters within a competition vial, however, I soon discovered that in addition to fecundity issues, the GFP strains
now show reduced survivorship which was not evident in these same strains in Fitzpatrick et al. (2007). Survivorship to pupation (index of fitness) was therefore calculated only for the unmarked flies which were then compared to each other. As a control I also seeded vials with either 32 unmarked rovers or 32 unmarked sitters. I used a Two-Way ANOVA to assess the role of genotype (rover, sitter) and nutrient abundance (low- and high-nutrient) on fitness and found a significant effect of genotype (p = 0.05), nutrient abundance (p < 0.0001) and the interaction term (p = 0.02)(F_{3,76} = 11.57, p < 0.0001). To assess this further I then conducted t-tests on the two nutrient abundances and found that rovers had higher fitness (0.86 ± 0.02, N = 20) than sitters (0.79 ± 0.02, N = 20) on low-nutrient food (Student’s t = 2.98, df = 38, p = 0.005). However, the survival of rovers and sitters was statistically indistinguishable when reared on high-nutrient food (rover: 0.90 ± 0.02, N = 20, sitter: 0.90 ± 0.02, N = 20) (Student’s t = 0.29, df = 38, p = 0.77). In the competition treatments under low-nutrient conditions, my analyses considered two factors: genotype (rover, sitter) and ratio (84:16 and 16:84 of rover: sitter). The overall Two-Way ANOVA was significant (F_{3,60} = 6.54, p = 0.0007) where I found a significant effect of genotype (p < 0.0001) but neither ratio (p = 0.41) nor the interaction term (p = 0.79)(Figure 5). Finally I conducted a t-test to assess the fitness of rovers and sitters and found that regardless of ratio, rovers had significantly higher fitness than sitters (rover: 0.92 ± 0.01, N = 32, sitter: 0.78 ± 0.14, N = 32) when competed in low-nutrient conditions (Students t-test, t = 4.39, df = 62, p < 0.0001). In summary, rovers have higher fitness than sitters under low-nutrient rearing conditions. This is maintained whether rovers are reared in the absence of genotypic competition (i.e. controls) or under conditions where the genotypes compete in ratios reflective of egg-laying preferences (i.e. competition experiments).
SUMMARY

The results from the above outlined experiments strongly suggest a role of the foraging gene in the egg-laying behaviour of *D. melanogaster*. Rover flies lay a significantly greater proportion of their eggs on low-nutrient substrates where sitters and the sitter mutant (*for*<sup>s2</sup>) show significantly higher preference for high-nutrient substrates. A transgenic manipulation also provides strong evidence that *for* is involved in oviposition site selection. When *for* expression in sitter flies is increased to rover-like levels, rover egg-laying preferences are rescued. By testing oviposition preferences of rovers and sitters in the presence of rover and sitter eggs, we simulated natural conditions. Under these conditions, there was no change in the egg-laying preferences of rovers and sitters. This suggests that the rover and sitter preferences observed in the lab may be similar to those expressed in the wild. Data also suggests that rover larvae show higher fitness than sitters when reared alone and when in competition with sitters on low-nutrient substrate, but there is no difference between genotypes either alone or under competition when reared on high-nutrient substrate.
4. Discussion

The list of genes known to affect complex behaviour is increasing (Sokolowski 2001; Bucan and Abel 2002; Anholt and Mackay 2004; Fitzpatrick et al. 2005; Mackay and Anholt 2006; Mackay and Anholt 2007; Kent et al. 2008; Robinson et al. 2008), where many such behaviours are known to have significant fitness implications. The ability to identify the individual genes important in regulating behaviour can not only answer proximate hypotheses about the mechanisms involved, but can also answer evolutionary questions regarding species relatedness and how populations evolve.

By using the naturally occurring rover/sitter polymorphism, this thesis explores the link between the foraging gene and oviposition and attempts to begin to dissect any fitness-related effects of egg-laying preferences of rovers and sitters.

The role of for in oviposition site selection in Drosophila melanogaster

Although empirical studies suggest a genetic influence of egg-laying behaviour (Takamura 1980; Takamura and Fuyama 1980; Kamping and van Delden 1990), the genetics of egg-laying preferences have been largely unexplored. Of the genes known to affect oviposition, only one (Gr5a) has been shown to affect substrate preferences (Yang et al. 2008). Yang et al. (2008) utilized a transgenic manipulation similar to those used in Experiment 2 to demonstrate rescued oviposition preferences in adult female D. melanogaster when Gr5a was overexpressed. Similar to a common lab stock, these flies were shown to prefer a more bitter (lobeline) than sweet (glucose) oviposition substrate. Aside from Gr5a, other genes have been shown to influence egg-laying behaviour, but not preference. Work on egg-laying rhythms in D.
*melanogaster* supports the idea that a network of circadian clock genes (e.g. *period*, *timeless*, *clock* and *cycle*) may govern the 24 hour egg-laying cycle observed in flies (reviewed in Manjunatha *et al.* 2008). More specifically, PER and TIM proteins are found to be highly expressed in ovarian tissue (Plautz *et al.* 1997; Hardin 2005), giving strong evidence for the importance of the circadian clock genes in egg-laying behaviour. In *C. elegans*, researchers employing a ‘bottom-up’ approach discovered 40 genes affecting a suite of egg-laying behaviours and morphology effecting egg-laying (Trent *et al.* 1983). Most of the genes described by Trent *et al.* (1983) fall under one category known as *egg-laying abnormal* (*egl*). Mutations in these genes causes a wide range of effects ranging from abnormal vulva development (*egl-16, egl-26, egl-29*) to releasing few or no progeny (*egl-23* and *egl-38*) (Trent *et al.* 1983), however, as all of these mutations were laboratory-induced, questions remain regarding the role of natural variation in these *egl* genes on egg-laying behaviour and/or preferences. In the present study I used a candidate gene approach to determine a genetic basis of oviposition preferences. Since oviposition in *D. melanogaster* contains elements of foraging behaviour, natural variants for larval and adult foraging behaviour, rovers and sitters, were assayed for differential egg-laying preferences. The differences observed between rovers and sitters for egg-laying preferences, combined with two genetic manipulations (mutant and transgenic over-expression) provides compelling evidence that naturally occurring allelic variation in *foraging* affects oviposition site selection behaviour.

Recently, numerous studies have identified numerous pleiotropies associated with allelic variation in *foraging*. Initially described to influence larval foraging behaviour (Sokolowski 1980; deBelle *et al.* 1989) it is now known that variation in *for* also influences adult foraging (Pereira and Sokolowski 1993), learning and memory performance in larvae and adults (Kaun *et
al. 2007b; Mery et al. 2007), pupation site selection (Sokolowski 1985; Rodriguez et al. 1992) sucrose responsiveness and habituation (Belay et al. 2007; Scheiner et al. 2004), food intake and energy homeostasis (Kaun et al. 2007a), the physiology of the neuromuscular junction (Renger et al. 1999), and neuroprotection during heat and hypoxic stress (Dawson-Scully et al. 2007; 2010). 

*foraging* encodes a cGMP-dependent protein kinase (PKG) and this vast assortment of pleiotropic effects associated with *for* is likely facilitated by the diverse role of PKG at the molecular and cellular level (Hofmann 2005; reviewed in Reaume and Sokolowski 2009; see above)

In addition to influencing multiple phenotypes, *foraging* shows conserved gene function across numerous taxa including two species of ants, honeybees and nematodes (reviewed in Reaume and Sokolowski 2009; see above). The orthologue of *for* in the nematode *C. elegans* (*egl-4*) shares many of the same pleiotropic effects as *for*. However, *egl-4* is included in the list of *egg-laying abnormal* mutants mentioned above. Since *egl-4* and *for* share many similar pleiotropic effects and egg-laying in *D. melanogaster* contains elements of foraging behaviour (Yang et al. 2008), it was hypothesized that *for* also effects egg-laying behaviour in the fruit fly. Results from my study indeed show an influence of *for* on egg-laying behaviour in *D. melanogaster*. Rovers prefer to deposit eggs a significantly greater proportion of their eggs on a low-nutrient substrate, than sitters and the sitter mutant. Preferences of flies that have increased *for*-mRNA and thus increased *for*-PKG also show rover-like preferences.

Since PKG is a major signalling molecule, the large list of food-related behaviours it influences in *D. melanogaster* was not entirely surprising after initially being discovered. Indeed, previous work by Fitzpatrick and Sokolowski (2004) suggest a common evolutionary origin for food-related PKG affected behaviours. What is more interesting is the vast array of behaviours
that have been related to PKG across multiple taxa. PKG is also known to modulate such
behaviours as the already described foraging behaviour in *D. melanogaster, C. elegans, Phediole
pallidula, Pogonomyrmex barbatis and Apis mellifera*, circadian rhythmicity in *D.
melanogaster, Mus musculus* and *Rattus norvegicus* and stress tolerance in *D. melanogaster* and
*M. musculus* (reviewed in Reaume and Sokolowski 2009). It is clear from the above list that
PKG serves as a major modulator of behaviour. Work on the common lab mouse *M. musculus*
provides the closest framework for how we expect PKG to influence behaviour in other
vertebrates. In the mouse, PKG has been shown to influence cocaine and alcohol addiction
behaviour (*Werner et al. 2004*) as well as anxiety (*Jouvert et al. 2004*). In the lab rat *R.
norvegicus* PKG has been shown to affect the progression of night to day behaviour (*Tischkau et
al. 2004*). In the fruit fly, PKG has been shown to effect carbon uptake and food utilization
(*Kaun et al. 2007*). Currently the link from PKG to human behaviour is under explored;
however, drawing from these previous studies, it is likely that PKG is a major modifier of
important human behaviours as well.

**Fitness consequences of rover and sitter egg-laying preferences**

According to my results, rover flies appear to significantly prefer a sub-optimal food
substrate upon which to deposit eggs. The low-nutrient substrate used in this experiment is
known to have a significant impact on fitness as evidenced by a significantly increased
development time, smaller third instar body size, and reduced survivability relative to the high
nutrient substrate (*Kaun et al. 2007a, Fitzpatrick et al. 2007, this study*). Two hypotheses to
explain this response in rovers includes: i) higher fecundity, and ii) better nutrient absorption and
metabolism. In my experiments I found that rovers laid approximated 2.8 times as many eggs as
sitters whereby the lower survivorship associated with the low-nutrient substrate may be offset in rovers by their increased fecundity. Secondly, work by Kaun et al. (2007a) has shown that rovers are better able to absorb carbon from glucose than sitters despite the fact that rovers consume less food than sitters during a given period of time. Indeed, results from Kaun et al. (2007a) and this study show that rovers reach higher rates of pupation than sitters when reared on low-nutrient food, but there is no difference in rates of pupation when reared on high-nutrient food. It is therefore possible that the negative fitness effects of ovipositing on low-nutrient substrate may be avoided by the more efficient rover metabolism.

Recent work by Yang et al. (2008) provides strong evidence that female D. melanogaster assess possible oviposition substrate and make a choice of substrate based on a simple decision making process. In my study, it is clear that rovers are choosing to deposit a higher proportion of their eggs on a low-nutrient substrate. The reasons and evolutionary implications of this choice are still unclear aside from the hypotheses described in the preceding paragraph. However, this preference does not change when another individual’s eggs are present on the food substrate. Early work with rovers and sitters show that rovers not only move longer distances within a food patch while feeding, but are more likely to leave that food patch in search of another (Sokolowski 1980; Pereira and Sokolowski 1993). It is possible that because of this increased searching behaviour, the risk of laying a high proportion of eggs on one poor food source is decreased. In nature, if rover and sitter larvae encounter an extremely poor food source, rovers would appear likely to leave, search and locate a different, possibly more nutritious food source. Data from this study on the number of eggs laid supports the hypothesis that rovers, more than sitters, are better able to recover from any risk-taking involved with larval foraging behaviour.
In the natural world rovers and sitters coexist, where they share the same food and oviposition substrates (Sokolowski 1980), therefore tests of fitness when in the presence of the same and the alternate genotype are clearly important. Results from my study suggest that rover larvae reared on low-nutrient food reach pupation at greater rates when reared with other rovers. Rovers also reach higher rates of pupated when reared on low-nutrient food when competing with sitters for resources. This is evident when rovers and sitters are reared in proportions at a three-to-one ratio (Fitzpatrick et al 2007) or when reared in proportions similar to the observed egg-laying preferences on low-nutrient media (this study). An explanation for this fitness difference between rovers and sitters comes from the work mentioned above. Rover flies have higher glucose absorption into the gut than sitters (Kaun et al. 2007a). The ability of rovers to exploit a nutrient poor resource effectively may outweigh any fitness costs associated with larval competition on low nutrient food.

The decision of where to lay one’s eggs can have significant fitness consequences. While it appears that rovers are choosing a sub-optimal food substrate upon which to deposit eggs, it also appears that independently of sitters, rovers have evolved to exploit this food source with minimal fitness consequences. While the exact reasons for the development and maintenance of this rover ability is currently unknown, it leads in many future research directions.

**Future Work**

The implications for fitness of the observed egg-laying preferences of rovers and sitters have not yet been fully explored. Although survivorship differences on low-nutrient substrates have been documented, this is not the only measure of fitness. Future work should test for effects of larval diet on mating behaviour. This could include mate searching, courtship, copulation
duration, sperm viability and numbers, as well as any sexually antagonistic behaviour between the sexes. The ability to pass one’s genes on to the next generation is important for an individual’s fitness and survivorship (as measured above) is only a small, albeit important player in the ability to parent offspring.

Previous work with *D. melanogaster* has successfully induced rover-like stress tolerance behaviour by exposing sitters to the PKG activator 8-Br-cGMP (Dawson-Scully *et al.* 2007), which activates pre-existing PKG in the cell. However, attempts at using this protocol for oviposition behaviour have been unsuccessful (see Appendix 4). Since this method has been previously successful, it may be useful to collaborate with researchers capable of measuring PKG activity levels to assess the timing of the decay in elevated PKG levels. As peak oviposition occurs in the early evening, it would be interesting if the observed rover and sitter oviposition preferences were due to spikes in PKG activity during this time. Determining the rate of decay and/or utilization of PKG could have important implications in the modulation of PKG influenced behaviour.

Although the usefulness of *D. melanogaster* in the laboratory setting is clearly visible, what are still lacking are studies of natural history. A good test of oviposition preferences in the field could collect fruits containing eggs. These eggs could then be reared in the lab until which each fly can be identified by molecular genotyping. This study would link a laboratory study on egg-laying preferences to what is happening in the natural setting.

Finally, future work can test for differences in taste receptors between rovers and sitters. Current work in the lab has began to tease apart the specific food component cues which drive egg-laying preferences and is alluding to different food components effecting rover and sitter choices. Yang *et al.* (2008) suggests a possible link between oviposition and the sweet taste
receptor $Gr5a$. There are methods available including the GAL4/UAS system in $D.\ melanogaster$ which allows for the activation or hyper-polarization of such taste receptors. Work can include looking at staining patterns of for-PKG to see if the $Gr5a$ neurons occur in the same locations in the nervous system. Also, use of transgenics with rovers and sitters could be used to test for any differences between the importance of taste receptors in egg-laying preferences. Work on taste receptors could potentially begin to answer the more mechanistic questions regarding the basis of egg-laying preferences.

**Conclusion**

My study provides evidence that the *foraging* gene is important for oviposition preferences in *Drosophila melanogaster*. Rover females lay a significantly greater proportion of eggs on low-nutrient food when compared with sitters. This was first verified by assaying the egg-laying preferences of the sitter mutant which displayed similar egg-laying preferences as sitters. Transgenic crosses using the pan-neuronal driver *elav*-GAL4 to overexpress for-mRNA in sitter flies to rover-like levels also provides strong evidence. Indeed, when for-mRNA is increased in sitters, their oviposition preferences changes to that of a rover. Also, this thesis shows that the egg-laying preferences observed between rovers and sitters seem to match the different phenotypes ability to utilize different quality food sources and thus reduce fitness costs. By dissecting oviposition behaviour in *D. melanogaster* this thesis examines the mechanisms underlying observed egg-laying preferences as well as describing relevant ecological reasons as to why such differences can be maintained in nature. This thesis aims to combine behavioural genetics and behavioural ecology, and in so doing, begins to weave answers to important biological questions which may be relevant to a large array of taxa.
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6. Tables and Figures

Table 1. The number of eggs per genotype placed on oviposition food patches per treatment. Eggs were placed on oviposition food patches to simulate a previously used environment. The breakdown of the number of eggs placed on either high- or low-nutrient food was devised from the egg-laying preferences of rovers and sitters (Figure 1).

<table>
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<tr>
<th>Treatment</th>
<th>Number of eggs per genotype pre-seeded onto food patches</th>
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<td></td>
<td>high-nutrient</td>
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<tr>
<td>rover eggs, &amp; pattern</td>
<td>14</td>
</tr>
<tr>
<td>rover eggs / sitter pattern</td>
<td>26</td>
</tr>
<tr>
<td>sitter eggs / rover pattern</td>
<td>-</td>
</tr>
<tr>
<td>sitter eggs &amp; pattern</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2. The number of larvae used per genotype per treatment to test for fitness consequences of the observed oviposition preferences of rovers and sitters (Figure 1.) GFP-tagged flies were utilized to aid in differentiating between genotypes. The ratio of larvae used in each genotype was calculated to reflect observed oviposition preferences and differences in fecundity between rovers and sitters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of larvae per genotype used in each treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rover larvae</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>rover-GFP : sitter</td>
<td>-</td>
</tr>
<tr>
<td>rover : sitter-GFP</td>
<td>27</td>
</tr>
<tr>
<td>sitter : rover-GFP</td>
<td>-</td>
</tr>
<tr>
<td>sitter-GFP : rover</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 1. The proportion of eggs laid on high- and low-nutrient substrates by rovers (*for*<sup>R</sup>), sitters (*for*<sup>s</sup>) and the sitter mutant (*for*<sup>s2</sup>). Rovers lay a significantly greater proportion of their eggs (0.66 ± 0.02, mean ± s.e.m) on low-nutrient substrates than both sitters (0.31 ± 0.02) and the sitter mutant (0.34 ± 0.02) (One-Way ANOVA, F<sub>2,117</sub> = 56.30, p < 0.0001, Tukey post hoc). N = 20 trials per genotype.
Figure 2. The proportion of eggs laid on high- and low-nutrient substrates by transgenic flies. Sitter flies with transgenically increased for-mRNA expression to rover-like levels (elav-GAL4/UAS-forT2) laid a greater proportion of eggs on low-nutrient food (0.81 ± 0.07) than two control crosses: elav-GAL4/+ (0.41 ± 0.04) and +/UAS-forT2 (0.40 ± 0.05) (One-Way ANOVA, $F_{2,60} = 16.72$, $p < 0.0001$, Tukey post hoc). N = 20 trials per cross.
Figure 3. The proportion of eggs laid on high- and low-nutrient substrates by rovers, sitters and the sitter mutant with rover or sitter eggs already present in either a rover or sitter egg-laying pattern. When in the presence of other eggs, rovers still lay significantly more eggs on low-nutrient substrates across all treatments than sitters and the sitter mutant (One-Way ANOVA, $F_{2,237} = 91.71, p < 0.0001$, Tukey post hoc). $N = 20$ trials per genotype per treatment.
**Figure 4.** The proportion of rovers and sitters that survived to pupation (fitness) when reared on low- and high-nutrient substrates. Rovers achieve higher fitness (0.85 ± 0.02 s.e.m) than sitters (0.78 ± 0.02) when reared on low-nutrient food whereas rover and sitter fitness on high-nutrient food are indistinguishable (Two-Way ANOVA, $F_{1.76} = 24.79$, $p < 0.0001$). $N = 20$ trials per genotype.
Figure 5. Proportion of rover and sitter larvae surviving to pupation (fitness) when reared with the alternate genotype in low-nutrient substrate at 84% or 16% of the total population. When reared with sitters, rovers reach a higher percentage of pupation at 16% (0.93 ± 0.03) and 84% (0.91 ± 0.01) of the total population than sitters at 16% (0.80 ± 0.05) and 84% (0.77 ± 0.02) (Students t-test, t = 4.39, df = 62, p < 0.0001), where there was no significant effect of rover/sitter ratio. N = 16 trials per genotype per treatment.
Appendix 1. Replicating preliminary data

Summary

Preliminary evidence supported the hypothesis that foraging influenced oviposition preference. However, this work completed during the summer of 2008 at The University of Toronto Mississauga was not complete. By replicating these preliminary findings during the winter of 2008-09, similar rover and sitter trends were found, however, a decrease in egg laying was discovered (Table A1.)

Methods

All methods used to complete this experiment were identical to those described for oviposition preference testing (see main text), except for flies were five days old when tested.

Results

Table A1. A replication of preliminary work showing a discrepancy in the number of eggs laid by rovers and sitters where higher numbers of eggs were observed in preliminary trials (UTM) than at UTSC.

<table>
<thead>
<tr>
<th>Location</th>
<th>Genotype</th>
<th>Mean Number of eggs</th>
<th>Standard error</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>for&lt;sup&gt;R&lt;/sup&gt;</td>
<td>134.1</td>
<td>20.84</td>
<td>20</td>
</tr>
<tr>
<td>UTM</td>
<td>for&lt;sup&gt;s&lt;/sup&gt;</td>
<td>66.4</td>
<td>15.40</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>for&lt;sup&gt;R&lt;/sup&gt;</td>
<td>43.9</td>
<td>8.75</td>
<td>42</td>
</tr>
<tr>
<td>UTSC</td>
<td>for&lt;sup&gt;s&lt;/sup&gt;</td>
<td>5.9</td>
<td>0.95</td>
<td>55</td>
</tr>
</tbody>
</table>
Appendix 2. Determining optimal egg-laying age

Summary

Work to correct the differences in egg-laying included testing flies at different ages. Having more eggs per trial, would lead to stronger results, and more accurate statistical analyses. The table below shows the difference in mean number of eggs laid by 5 and 3 day old flies.

Methods

All methods used to test for optimal egg-laying age are identical to those described above in the main text. Flies were tested at ages ranging from 2-7 days old.

Results

Flies laid the greatest number of eggs per trial when three days old (Table A2. Data from ages 2, 4, 6, 7 not shown)

Table A2. Troubleshooting to determine the best age for egg-laying. It was found that the most eggs were laid by 3 day old flies across all genotypes (ages 2,4,6, and 7 not shown).

<table>
<thead>
<tr>
<th>Fly age (d)</th>
<th>Genotype</th>
<th>Mean number of eggs</th>
<th>Standard error</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>for^R</td>
<td>66.3</td>
<td>5.031</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>for^s</td>
<td>23.6</td>
<td>3.007</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>for^a2</td>
<td>32.2</td>
<td>2.535</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>for^R</td>
<td>43.9</td>
<td>8.749</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>for^s</td>
<td>5.9</td>
<td>0.946</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>for^a2</td>
<td>24</td>
<td>5.704</td>
<td>5</td>
</tr>
</tbody>
</table>
Appendix 3. Transgenic manipulation using *heat-shock* (*hs*) as a GAL4 driver

**Summary**

Rovers differ in their PKG activity and levels than sitters. One previously successful method of rescuing rover-like behaviour in sitter flies by increasing *for*-mRNA leading to increased *for*-PKG is by the use of transgenics. Transgenics allows us to target gene expression, and over-express our gene of interest.

Transgenic flies were previously generated and crossed into a *for*\(^s\) background, creating *w*\(^1\); *for*\(^s\); *UAS-for*\(^T2\) (Belay et al. 2007). Furthermore, the transgenic driver line was also crossed onto sitter genetic background: *w*\(^1\); *for*\(^s\); *hs-GAL4* (Osborne et al. 1997). *hs* represents a temperature-sensitive promotor that is expressed naturally at low levels throughout the organism (in absence of stress, called leaky expression, e.g. 18-25 °C) but this expression increases drastically under extreme stress (e.g. heat shock, >30 °C). We can capitalize on the low level *hs* expression at 23 °C, to overexpress *for*\(^T2\) throughout the fly. This over-expression leads to rover-like expression levels and behaviour in sitters (Belay et al. 2007).

**Methods**

The protocol for this experiment was identical to that shown for the transgenic manipulation described in the main text above, however, the fly strain *w*\(^1\); *for*\(^s\); *hs-GAL4* was substituted for *w*\(^1\); *for*\(^s\); *elav-GAL4*. 
**Results**

Results show a severe decrease in egg-laying in the experimental cross (Table A3).

Having very little egg-laying, as well as many trials with no eggs laid, led to inaccurate and weak statistical analysis.

**Table A3.** Data showing mean number of eggs laid utilizing a transgenic manipulation using the *heat-shock (hs)* GAL4 driver. This test resulted in a decrease in egg-laying in the experimental cross.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean number of eggs</th>
<th>Standard Error</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w^1; for^5; hs\text{-}GAL4 / + )</td>
<td>30.6</td>
<td>2.26</td>
<td>20</td>
</tr>
<tr>
<td>( w^1; for^5; + / UAS\text{-}forT2 )</td>
<td>15.99</td>
<td>1.44</td>
<td>73</td>
</tr>
<tr>
<td>( w^1; for^5; hs\text{-}GAL4 / UAS\text{-}forT2 )</td>
<td>2.23</td>
<td>0.46</td>
<td>43</td>
</tr>
</tbody>
</table>
Appendix 4. Pharmacological manipulation to rescue rover-like behaviour in sitters

Summary

In the cell, PKG is activated by cyclic guanosine monophosphate (cGMP). The PKG molecule has two cGMP-specific binding sites and PKG remains inactive until cGMP binds at these sites (Kalderon and Rubin 1989). As described above, rovers and sitters differ in their levels of active PKG where rovers have higher activity levels. By providing an exogenous source of cGMP (8-Br-cGMP, Sigma), we can artificially increase PKG activity levels in sitters that lead to rover-like PKG activity levels and behaviour. For example, studies recently found that a one hour exposure of sitter flies to 10 mM 8-Br-cGMP was sufficient to induce to rover-like behavioural responses to anoxic stress (Dawson-Scully et al. 2010). Preliminary work looked promising in rescuing rover-like egg-laying behaviour in sitter flies. Based on these results, I proposed to utilize a similar protocol as that used by Dawson-Scully et al. (2010) to manipulate OSS in sitters.

Methods

Methods for the assaying of OSS are identical to those described in the above experiments, however, flies were 5 days old when assayed, and were pre-treated with chemical agents as outlined by Dawson-Scully et al. (2010). Sitters were exposed to 10 mM 8-Br-cGMP (B1381, Sigma) that is solubilized in dimethyl sulfoxide (DMSO; D8418, Sigma). Additionally, an exposure to DMSO itself was used as a sham control. Batches of 10 females were placed into exposure vials. Exposure vials were prepared by first adding 10 µL of the chemical solution to a Kim-Wipe that was crushed at the bottom of the vial. An additional crushed Kim-Wipe was added over top of this to prevent direct contact between the flies and the solution. The vial was
capped with a buzz plug stopper and then the finger from a latex glove was stretched over the top to contain the vapours. Flies were exposed to the chemicals for 1 hr in the dark. Six exposure vials were combined into one oviposition assay (i.e. 60 females). This methodology, exposing batches of 10 females, has been shown to significantly increase PKG activity in sitters to rover-like levels (Dawson-Scully et al. 2010)

Results

Results show an effect on egg-laying preference in rover and sitter flies. Unexpectedly, rover and sitter preference for low-nutrient food decreased with chemical treatments.
Figure A4a. The proportion of eggs laid on low-nutrient food by rovers (for^R) and sitters (for^s) in three treatments. Black bars represent a control treatment where no chemical was administered. Grey bars represent flies exposed to the PKG activator 8-Br-cGMP. Blue bars represent a DMSO control. Both chemical treatments resulted in a decrease in number of eggs laid on low-nutrient food by both rover and sitter flies. All tests conducted in March 2009. N = 3 Trials each for rover 8-Br-cGMP and DMSO control treatments, N = 5 for sitter 8-Br-cGMP and N = 2 for sitter DMSO control treatments respectively.
Replication of Pharmacology manipulation with 3 day old sitter flies

Figure A4b. A replication of previous pharmacology data but instead with 3 day old flies. Results show the proportion of eggs laid on low- and high nutrient food by sitters when exposed to the PKG activator 8-Br-cGMP. After being exposed, no change in preference is observed in sitter flies. All tests conducted in conjunction with data presented in Figure 1 of main text. N = 11 trials.
Appendix 5. Testing rover/sitter egg-laying preferences when in the presence of another genotype

Summary

In nature, rovers and sitters co-exist. However, oviposition assays described in experiment 1 and 2 test genotypes in isolation. To achieve better understanding of egg-laying preferences in the natural world, assays including both rovers and sitters together are needed.

Methods

Oviposition assays were conducted in an identical fashion to those described above. However, to differentiate between rovers and sitters when tested together, I utilized the GFP marker. Flies were tested in groups of 64 where the proportion of rovers to sitters was reflective of proportions of rovers and sitters observed in the wild (3 rovers: 1 sitter) (Sokolowski 1980). Two control ratios (1:1, and 1:3 rovers: sitters) were also established. After which, it was planned that the oviposition food caps (low- and high-nutrient) with rover and sitter eggs would be placed in a fly vial with fly food of the same nutrient abundance for larval rearing.

Results

It was discovered that flies tagged with GFP showed a decrease in fecundity making further larval rearing impossible.
Table A4. The number of eggs laid by rover, rover-GFP, sitter and sitter-GFP in oviposition assays where genotypes were combined. In these trials, rovers were paired with sitter-GFP and rover-GFP with sitters at ratios of 3:1, 1:1 or 1:3.

<table>
<thead>
<tr>
<th>Number of flies tested</th>
<th>Number of eggs laid per genotype</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rover eggs</td>
<td>sitter eggs</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>S.E.</td>
</tr>
<tr>
<td>16</td>
<td>30.1</td>
<td>5.9</td>
</tr>
<tr>
<td>32</td>
<td>55.8</td>
<td>8.0</td>
</tr>
<tr>
<td>48</td>
<td>41.5</td>
<td>8.1</td>
</tr>
</tbody>
</table>